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MOST early-onset familial Alzheimer disease is associated with missense mutations in S182, a membrane protein on chromosome 14. We investigated amyloid- $\beta$  protein (A $\beta$ ) precursor (A $\beta$ PP) metabolism in skin fibroblasts from S182 (Glu $^{246}$ )-affected individuals and unaffected family members. Steady-state A $\beta$ PP levels were similar among all lines as was the degree of increase in soluble A $\beta$ PP released upon stimulation of cells with either phorbol ester or serum. Among all lines studied, A $\beta$  levels were consistently detectable only in the medium of a single line of S182 (Glu $^{246}$ ) cells, consistent with the conclusion that some S182 mutant lines may accumulate A $\beta$  in their conditioned media. Studies of cells from additional individuals and under other conditions will be required to establish this association of elevated A $\beta$  levels with S182 mutations.

**Key words:** Alzheimer's disease; S182; Amyloid- $\beta$  protein precursor; Glu $^{246}$

## High levels of amyloid- $\beta$ protein from S182 (Glu $^{246}$ ) familial Alzheimer's cells

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### Introduction

Sporadic and familial forms of Alzheimer's disease (AD) are characterized clinically by progressive dementia and pathologically by defective signal transduction and by accumulation in brain of abnormal protein structures, the most disease-specific of which are extracellular deposits composed primarily of the amyloid- $\beta$  protein (A $\beta$ ; for review see Refs 1, 2). In rare forms of early-onset familial AD (FAD), missense mutations in the A $\beta$  precursor (A $\beta$ PP) segregate with the FAD phenotype and appear to cause AD by generation of excess<sup>3,4</sup> or extended<sup>5</sup> A $\beta$  peptides. In some examples of A $\beta$ PP-mutant FAD, abnormalities in A $\beta$ PP metabolism can be demonstrated in cultured skin fibroblasts from biopsy of A $\beta$ PP-mutation-bearing individuals.<sup>6,7</sup> Most (~70–80%) cases of early-onset FAD are associated with anonymous genetic markers on chromosome 14<sup>8–11</sup> and have recently been demonstrated to segregate with missense mutations in a membrane protein,<sup>12</sup> designated S182. Though the function of S182 is not immediately obvious from its primary structure, it bears some homology to molecules involved in vesicle trafficking and other homologies to molecules involved in signal transduc-

tion.<sup>12</sup> Since both of these putative cell biological functions of S182 can modulate A $\beta$ PP metabolism,<sup>1</sup> and since independent evidence has linked signal transduction abnormalities to clinical AD phenotype in cultured skin fibroblasts from sporadic and familial AD patients,<sup>13–22</sup> including those with S182 mutations, we investigated basal and regulated A $\beta$ PP metabolism in cultured skin fibroblasts from S182 (Glu $^{246}$ ) mutant and unaffected individuals.

Steady-state A $\beta$ PP levels were similar among all fibroblast lines studied as was the degree of increase in soluble A $\beta$ PP (sA $\beta$ PP) released upon stimulation of the fibroblasts with either phorbol ester or serum. Thus, stimulated sA $\beta$ PP release is apparently normal in S182 (Glu $^{246}$ ) skin fibroblasts. Among all lines studied, the highest A $\beta$  levels were detected in the medium of one line of S182 (Glu $^{246}$ ) mutant cells, supporting the possibility that basal A $\beta$  levels are elevated in medium conditioned by skin fibroblast lines from S182 mutant individuals, as has been recently described in separate published<sup>23</sup> and reported<sup>24</sup> investigations. Studies of additional cell lines and experimental conditions will be required to establish this association of S182 mutations with elevated A $\beta$  levels in the medium of cultured skin fibroblasts.

## Materials and Methods

**Reagents:** Phorbol-12,13-dibutyrate was purchased from Sigma Chemical Co (St. Louis MO). Affinity-purified anti-695 APP<sup>WT</sup> was prepared as described.<sup>25</sup> Agarose-coupled anti-mouse and anti-rabbit secondary antibodies were purchased from HyClone (Logan UT). Protein A-Sepharose was obtained from Pharmacia LKB (Piscataway NJ). 10–20% Tris-tricine gradient gels and 10% tris-tricine gels were purchased from Novex (San Diego CA). [<sup>35</sup>S]methionine was purchased from New England Nuclear-Dupont (Boston MA).

**Cell lines:** Human skin fibroblasts were obtained from the NIA Aging Cell Repository at the Coriell Institute for Medical Research. Cells designated by Repository Numbers AG06848, AG06840, and AG08170, from the Canadian *FAD1* pedigree bearing the S182 (Glu<sup>246</sup>) mutation, were used. The ages of these individuals at the time of biopsy were 55, 56 and 56 years, respectively. All were diagnosed as affected by AD based on clinical criteria, and postmortem neuropathological examination of patient AG06848 confirmed the diagnosis of AD. In addition, DNA from line AG06848 was sequenced and the presence of the S182 (Glu<sup>246</sup>) mutation was confirmed (S. S. Sisodia, personal communication). In addition to the presence of the S182 mutation, additional criteria for this choice of cell lines included the existence of previously reported data indicating that these lines exhibited abnormalities in certain signal transduction assays,<sup>14–22</sup> since some of these signalling pathways have been implicated in the regulation of A<sub>β</sub>PP metabolism.<sup>1</sup>

Three cell lines from unaffected members of the *FAD1* kindred were chosen for study as controls. These cells were designated by Repository Numbers AG06842, AG06846, and AG07657, and were taken from individuals of 75, 75, and 88 years of age, respectively, at the time of biopsy.

**Metabolic labelling and studies of A<sub>β</sub>PP metabolism:** Cells were maintained as described.<sup>18</sup> The day before metabolic labelling was to be performed, cells were trypsinized and transferred to 6-well tissue culture plates. Cells ( $2 \times 10^6$ ) were seeded into each well in 2 ml Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Twenty-four hours later the medium was replaced with methionine-free medium and the cells were incubated for 45 min at 37°C prior to labelling. The cells were then incubated with 0.5 mCi (18.5 MBq) [<sup>35</sup>S]methionine in 1 ml of methionine-free DMEM. Metabolic labelling was carried out for either 20 min or 2 h, followed by incubation in complete, methionine-supplemented medium for 1–2 h prior to harvest. In each 6-well dish, cells treated for 0, 1, and 2 h were assessed in the

absence or presence of either phorbol or serum. These test compounds were added at the end of the metabolic labelling period. At the end of the incubation (0–2 h) the medium was removed and the cells were rapidly harvested. Cells and media were treated with 1% sodium dodecyl sulfate, boiled for 5 min, sonicated (lysates only), and centrifuged at 10 000 × g for 10 min. After dilution with an equal volume of neutralization buffer [6% (v/v) Nonidet P-40, 200 mM Tris-HCl, pH 7.4, 300 mM NaCl, 10 mM EDTA, 4 mM sodium azide], supernatants of standard protein concentration were incubated with primary antibodies overnight at 4°C (369A for lysates, 6E10 for media). Immune complexes were recovered using agarose-coupled anti-mouse IgG or with Protein A-Sepharose. Pellets were washed three times with 1 ml 0.5 M NaCl in Tris-buffered saline (100 mM Tris-HCl, 150 mM NaCl, pH 7.4), three times with TBS-Tween, and two times with TBS.

Samples containing immunopurified A<sub>β</sub>PP species were boiled in 30 µl of loading buffer [0.9 M Tris-HCl, pH 8.45, 24% glycerol, 8% SDS, 0.015% Coomassie Blue G and 0.015% phenol red] and separated by electrophoresis in premade commercial tris-tricine polyacrylamide gels (Novex; San Diego CA). Gels were treated with enhancer solution (Entensify; New England Nuclear-Dupont, Boston MA), dried, and quantified by phosphorimaging with a BioRad Molecular Analyst phosphorimaging system.

In some experiments, immunoblot analyses were performed as described.<sup>26</sup>

**A<sub>β</sub> assays:** A<sub>β</sub> in conditioned medium (72 h) was determined by 4G8/6E10 ELISA as previously described.<sup>27</sup>

**Statistical analyses:** Student's t-test and Wilcoxon matched pairs test were used for statistical analyses.

## Results and Discussion

**Identification of molecular forms of A<sub>β</sub>PP in human skin fibroblasts:** Human cultured skin fibroblasts synthesized readily detectable amounts of A<sub>β</sub>PP as evidenced by biosynthetic labelling and immunoprecipitation or by immunoblotting, using either antibody 369<sup>25</sup> or the A<sub>β</sub>PP-specific antibody 6E10.<sup>26</sup> An incompletely matured A<sub>β</sub>PP species of  $M_r \sim 100$  kDa was detected at the end of the metabolic labelling period. As expected based on A<sub>β</sub>PP metabolism in PC12 cells,<sup>25,28</sup> this immature A<sub>β</sub>PP diminished in prominence during the 2 h (post-metabolic labelling) incubation period as a slightly more slowly migrating ( $M_r \sim 110$  kDa) species, the putative fully mature A<sub>β</sub>PP species, appeared. As mature A<sub>β</sub>PP appeared, a C-terminally truncated species of  $M_r \sim 100$  kDa

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accumulated in the conditioned medium, corresponding to the putative soluble A $\beta$ PP or sA $\beta$ PP.

When assessed by immunoblotting analyses, a non-significant trend toward elevation of steady-state levels of cellular full-length A $\beta$ PP species was noted among the S182 (Glu $^{246}$ ) lines (Table 1).

**Stimulated sA $\beta$ PP release by phorbol ester and serum:** Numerous independent studies have documented in various cell types that activation of protein kinase C (PKC), via application of either phorbol esters<sup>25-29</sup> or certain first messengers,<sup>30,31</sup> is accompanied by a several-fold increase in sA $\beta$ PP release, a phenomenon which is known as 'regulated A $\beta$ PP cleavage' and which is at least partially explained by PKC stimulation of budding of new A $\beta$ PP-bearing transport vesicles from the trans-Golgi network.<sup>32</sup> In addition to, or perhaps because of, the regulated cleavage event leading to enhanced sA $\beta$ PP release, a concomitant diminution in A $\beta$  generation is typically observed.<sup>33-35</sup> Thus, integrity of the regulated cleavage process could be important for normal A $\beta$  homeostasis.

With regard to the current study, it is conceivable that if S182 participates in the regulated cleavage process (e.g. by contributing to the vesicle budding process, as proposed in one model<sup>12</sup>), then mutant S182 fibroblasts treated with either phorbol ester or serum (in order to activate PKC either directly or via first messenger-linked receptors, respectively) might exhibit a diminished fold-stimulation of sA $\beta$ PP release.

**Table 1.** Total Immunoreactive full-length A $\beta$ PP (arbitrary units) per unit protein in control or S182 (Glu $^{246}$ ) mutant cells. Means  $\pm$  s.d. for six replicate plates

Cell line	A $\beta$ PP
Control	
AG06846	81.2 $\pm$ 32.3
AG07657	69.3 $\pm$ 18.1
S182 Glu $^{246}$ mutant	
AG06848	94.4 $\pm$ 3.3
AG06840	98.3 $\pm$ 7.4
AG08170	88.8 $\pm$ 7.4

**Table 2.** Fold Increase in sA $\beta$ PP at 1 or 2 h following treatment of control or S182 (Glu $^{246}$ ) mutant cells with the indicated doses of phorbol-12,13-dibutyrate (PDBu). Similar results were obtained in 2-3 experiments with each cell line

	25 nM PDBu	50 nM PDBu	100 nM PDBu
Control			
AG06846 (1 h)	2.45	2.64	2.81
AG06845 (2 h)	2.43	5.10	6.45
AG07657 (1 h)	2.01	1.12	1.82
AG07657 (2 h)	1.76	1.62	1.52
S182 Glu $^{246}$ mutant			
AG06848 (1 h)	2.94	4.52	2.59
AG06848 (2 h)	1.78	4.52	4.24
AG06840 (2 h)	3.95	8.31	3.88
AG08170 (2 h)	3.00	2.98	2.66

**Table 3.** Increase in sA $\beta$ PP at 1 h following treatment of control or S182 (Glu $^{246}$ ) mutant cells with 10% fetal bovine serum. Data from two separate experiments are shown; N.T. = not treated

	Expt 1	Expt 2
Control		
AG06848	1.85	3.15
AG07657	N.T.	2.68
S182 Glu $^{246}$ mutant		
AG06840	2.23	2.44
AG08170	2.72	N.T.

**Table 4.** Summary of results of A $\beta$  ELISA of media conditioned for 72 h by control or S182 (Glu $^{246}$ ) mutant cells at indicated confluencies

	Confluency (%)	Protein ( $\mu$ g ml $^{-1}$ )	Cellular immunoreactive A $\beta$ PP (units)	Medium A $\beta$ ( $\mu$ g ml $^{-1}$ )
Control				
AG06846	100	20.2	26.36	17.21
	100	23.1	24.66	<10.0
	100	20.5	29.58	<10.0
	100	18.8	22.48	<10.0
	100	22.2	36.98	<10.0
	100	23.1	36.1	<10.0
S182 Glu $^{246}$ mutant				
AG06848	100	12.0	20.44	35.82
	80	10.9	20.42	26.11
	50	10.1	6.88	32.24
	70	12.7	10.03	28.26
	90	15.4	20.91	32.24
	85	16.4	26.6	18.44
AG06840	80	7.9	6.90	<10.0
	80	6.2	7.35	<10.0
	80	7.7	8.22	<14.5
	80	6.5	3.88	<10.0
	80	8.3	10.23	<10.0
	80	8.9	12.32	<10.0

However, in all lines studied, similar stimulation of sA $\beta$ PP release was observed in response to a range of doses of phorbol-12,13-dibutyrate (PDBu, 25–100 nM; Table 2) or to serum (Table 3). Stimulated sA $\beta$ PP release was accompanied by fractional diminution of the fully mature A $\beta$ PP species and was similar for all cell lines (not shown). Thus, PKC-regulated sA $\beta$ PP release is apparently normal in S182 (Glu $^{246}$ ) skin fibroblasts.

**A $\beta$  levels in fibroblast conditioned media:** Using metabolic labelling and immunoprecipitation method described above for assessing immature A $\beta$ PP levels, and a 4G8/6E10 ELISA<sup>27</sup> for measuring A $\beta$ , we assessed in parallel both the contemporaneous A $\beta$ PP synthesis by cells and the A $\beta$  levels in the respective conditioned media of one control (AG06846) and two FAD S182 mutant (AG06848 and AG06840) fibroblast lines grown to various confluencies.<sup>36</sup> The results, summarized in Table 4, indicate that conditioned medium from one S182 mutant line (AG06848) was consistently associated with the highest A $\beta$  level of all cell lines studied, consistent with other recent reports.<sup>23,24</sup> In the current study, this is of particular

note since this line (AG06848) was intermediate in its level of contemporaneous endogenous A $\beta$ PP synthesis compared with other lines, suggesting that cell line AG06848 might bear an intrinsic propensity toward high A $\beta$  generation or impaired A $\beta$  clearance, perhaps due to the S182 (Glu<sup>246</sup>) mutation. It is interesting to compare these results using S182 (Glu<sup>246</sup>) cell line AG06848 with results from another study<sup>23</sup> in which levels of A $\beta$  released by this line were the highest of all S182 (Glu<sup>246</sup>) lines depicted (see Ref. 23, Fig. 7). Since it is well recognized that human skin fibroblast data can be subject to wide interindividual and interlaboratory variations (compare Refs 14, 16, 37), we consider these confirmatory data worth noting.

This observation of increased A $\beta$  in the medium of a mutant S182 line, if frequently associated with mutant S182 in an extended study of control and mutant S182 lines, could reflect a fundamental property of the mutant S182 phenotype. Further, since A $\beta$ PP metabolism and A $\beta$  generation are controlled by a variety of factors including first and second messengers,<sup>1,24-35</sup> confluence,<sup>36</sup> cell cycle,<sup>38</sup> and state of differentiation,<sup>39</sup> it will be important to assess the impact of S182 mutations on A $\beta$ PP metabolism and A $\beta$  turnover under more standardized experimental conditions (e.g., studies of A $\beta$ PP and A $\beta$  in identical clonal cells following parallel transfection with either wildtype or mutant S182). The successful cloning of S182 and discovery of its pathogenic mutations now enables these investigations, and such experiments are underway.

## Conclusion

We investigated amyloid- $\beta$  protein (A $\beta$ ) precursor (A $\beta$ PP) metabolism in skin fibroblasts from S182 (Glu<sup>246</sup>)-affected individuals and unaffected family members. Steady-state A $\beta$ PP levels were similar among all lines as was the degree of increase in soluble A $\beta$ PP released upon stimulation of cells with either phorbol ester or serum. Among all lines studied, A $\beta$  levels were consistently detectable only in the medium of a single line of S182 (Glu<sup>246</sup>) cells, consistent with the conclusion that some S182 (Glu<sup>246</sup>) lines may accumulate A $\beta$  in their con-

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